CodeLink Gene Expression System: 16-Assay Bioarray Hybridization and Detection

Warning

For research use only. Not recommended or intended for the diagnosis of disease in humans or animals. Do not use internally or externally in humans or animals.

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CodeLink Expression Bioarray System

System description

CodeLink™ Expression Bioarray System comprises a set of bioarray products and tools for gene expression profiling experiments that allows monitoring of the mRNA levels of multiple genes simultaneously. The system includes:

- sets of carefully designed and validated bioarrays with integrated hybridization 16-assay chambers that cover a select group of discovery genes for several organisms
- target preparation and hybridization reagents
- optimized target preparation and processing protocols
- hybridization and bioarray processing tools
- bioarray analysis software

This user manual provides the protocol for the CodeLink 16-assay bioarray processing, including hybridization, post-hybridization processing, and scanning. The 16-assay bioarray format consists of 16 independent arrays and hybridization chambers in the space of a standard glass slide.

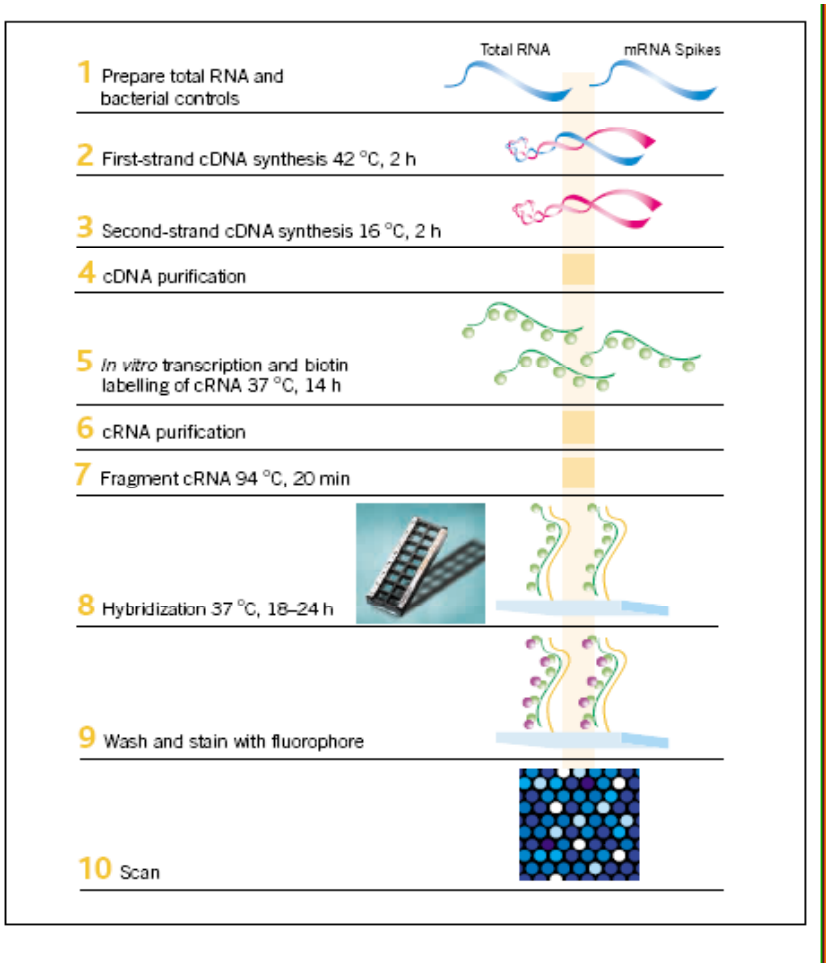

Total RNA
mRNA Spikes
1 Prepare total RNA and bacterial controls
2 First-strand cDNA synthesis 42 °C, 2 h
3 Second-strand cDNA synthesis 16 °C, 2 h
4 cDNA purification
5 In vitro transcription and biotin labelling of cRNA 37 °C, 14 h
6 cRNA purification
7 Fragment cRNA 94 °C, 20 min
8 Hybridization 37 °C, 18–24 h
9 Wash and stain with fluorophore
10 Scan

Fig 1. CodeLink Expression Bioarray signals are produced by the hybridization of biotin-labelled complementary RNA (cRNA) target(s) to DNA oligonucleotide probes attached to a gel matrix followed by secondary labelling and signal detection.

Safety warnings and precautions

Consider all chemicals as potentially hazardous. Only persons trained in laboratory techniques and familiar with the principles of good laboratory practice should handle these products. Wear suitable protective clothing such as laboratory coats, safety glasses, and gloves.
Exercise caution to avoid contact with skin or eyes; if contact should occur, wash immediately with water. See Material Safety Data Sheet for specific recommendations.

For each reagent required but not provided, follow the manufacturer's safety requirements.

Handling

These procedures involve working with RNA; therefore, exercise great care to avoid RNase contamination. All solutions must be RNase-free and pipette tips must be aerosol-resistant and changed before each step. Use commercially prepared nuclease-free water (rather than water treated with diethylpyrocarbonate (DEPC)) for all nucleic acid steps.

RNeasy™ is recommended for total RNA isolation.

Storage

CodeLink™ iExpress Assay Reagent Kit components should be stored as follows:

- hybridization buffer component A at ambient temperature.
- hybridization buffer component B and 5× fragmentation buffer at 4 °C.

The biotin-labelled cRNA target is prepared by a linear amplification method. The poly(A)+ RNA subpopulation (within the total RNA population) is primed for reverse transcription by a DNA oligonucleotide containing the T7 RNA polymerase promoter 5' to a d(T)24 sequence (Fig 1). After second-strand cDNA synthesis, the cDNA serves as the template for an in vitro transcription (IVT) reaction to produce the target cRNA.
The IVT is performed in the presence of biotinylated nucleotides to label the target cRNA. This method produces approximately 1000-fold to 5000-fold linear amplification of the input poly(A)+ RNA.

A set of bacterial control mRNAs is included in CodeLink iExpress Assay Reagent Kit as controls for the cDNA synthesis and the IVT reactions. These controls are added to the total RNA sample during target preparation. Each step of the CodeLink Expression Bioarray processing procedure, including target preparation and hybridization, can be monitored using these control mRNAs. Additionally, the bacterial control mRNAs can be used to estimate the sensitivity of RNA detection.

Hybridization is performed overnight in a temperature-controlled shaking incubator.
Optimized hybridization buffer components are also included in CodeLink Expression Assay Reagent Kit for use at this step.

Post-hybridization processing includes a stringent wash to remove unbound and non-specifically hybridized target molecules, a staining step with a Cy™5-Streptavidin conjugate, and several non-stringent washing steps to remove unbound dye conjugate.
Following a final rinse, the bioarrays are dried by centrifugation and scanned.
Analysis of the bioarrays with the CodeLink Expression Analysis software is described in the Help accessible in the software.

CodeLink Gene Expression System:

16-Assay Bioarray Hybridization and Detection

Protocol overview

Note: no overage is included in the amounts described in this Overview.

1. **Fragment cRNA.** 16 arrays require 96 µl total volume. For each array, bring 0.9 µg of cRNA to a final volume of 4.8 µl with water. Add 1.2 µl of 5× fragmentation buffer and incubate at 94 ºC for 20 minutes. Cool 5 minutes.

2. **Prepare hybridization reaction mixture.** Bring 6 µl of the fragmented cRNA solution, 18 µl of hybridization buffer component A, and 30 µl of hybridization buffer component B to a final volume of 60 µl with water. Incubate at 90 ºC for 5 minutes and immediately chill on ice for 5 minutes, then proceed to loading.

3. **Loading reaction mixtures into array chambers.** Slowly pipet the 60 µl hybridization reaction mixture into the middle of each array chamber taking care against splash contamination. Seal the entire 16-assay unit with a sealing strip.

4. **Hybridization.** Set the shaker speed to 225 rpm and incubate slides for 18–24 hours at 37 ºC, maintaining a consistent hybridization time for comparative experiments.

5. **Post-hybridization wash.** Carefully remove the 16-assay Chamber sealing strip. Flush each assay chamber three times with 250 µl of 0.75× TNT. Add 250 µl of 0.75× TNT to each assay chamber and seal entire 16-assay Chamber unit with a sealing strip. Transfer the 16-assay unit to a 46 °C oven and incubate for exactly 1 hour. Do not exceed 1 hour incubation.

6**. Detection with streptavidin-dye conjugate.** Remove the 16-assay Chamber unit from the oven and carefully remove the sealing strip. Aspirate the 0.75× TNT, keeping a small volume of fluid on the slide surface at all times. Add 250 µl of Cy5-Streptavidin for Microarrays working solution to each assay chamber. Place 16-assay Chamber unit in a dark area at ambient temperature for 30 minutes. Aspirate the Cy5-Streptavidin for Microarrays working solution after incubation.

7. **Wash.** Flush each assay chamber three times with 250 µl of 1× TNT. Add 250 µl of 1× TNT to each assay chamber and incubate at ambient temperature for 20 minutes. Aspirate the 1× TNT and add 250 µl of 0.1× SSC/0.05% Tween into each assay chamber. Immediately invert the 16-assay chamber unit and decant liquid into waste.

8. **Dry slides.** Quickly place the inverted chamber unit(s) into the slide drying tray and place into the centrifuge rotor bucket, with an absorbent paper towel at the bottom. Spin dry the Bioarrays. Separate the slide from the chamber unit by removing the unit clips with one hand while holding the associated slide with the other hand. Store dried bioarrays in the dark.

9. **Bioarray scanning and analysis.** Scan bioarrays using the 5-µm resolution setting and analyze with the CodeLink Expression Analysis software. Scanning with GenePix™ 4000B is described in this booklet. For use of CodeLink Bioarrays withother scanners, visit our website, www.appliedmicroarrays.com.

Use only high-quality cRNA generated with a CodeLink Expression Assay target preparation protocol for the following steps to achieve optimal results.

Avoid tip contact with the probe-containing area of each bioarray.

Exercise caution to avoid RNase contamination. All solutions must be RNase-free; pipette tips must be changed before each step. Use nuclease-free water for all nucleic acid steps. Use aerosol-resistant tips for all pipetting steps.

To avoid fluorophore photobleaching, prepare the Cy5-labelled Streptavidin conjugate in opaque or dark tubes. In addition, minimize bioarray exposure to ambient light, particularly after completing step 6.2.

Ensure that the ambient temperature in the laboratory is 23 ºC ± 2 ºC.

Minimize exposure to particulate matter. Clean all work areas prior to hybridization and analysis.

The list of reagents and equipment is recommended for best performance. Contact Applied Microarrays, Inc. technical support for information on use of alternatives.

Storage

Keep 5× fragmentation buffer at 4 ºC until use. Briefly vortex and spin down prior to use to ensure solution homogeneity.

Store hybridization buffer component A at room temperature. Since a precipitate may form upon storage, briefly vortex and spin down prior to use to ensure solution homogeneity. If necessary, warm at 37 ºC to redissolve.

Store hybridization buffer component B at 4 ºC until use.

For steps 1–2, use of a 10% volume mixture overage is recommended but not required.

cRNA should appear on the gel as a smear. If there is no smear, or if the smear is predominantly below 500 nucleotides, do not continue with the sample.

Products used for processing

CodeLink iExpress Assay Reagent Kit (Manual Prep, 24 reactions)	**67601000**
5× fragmentation buffer hybridization buffer component A hybridization buffer component B	
Cy5-Streptavidin for Microarrays (staining solution is a 1:500 dilution in TNB; see Appendix 1)	**28900224**
CodeLink Expression Analysis Software v5.0	**310035**
CodeLink Universal Shaker Kit	**310031**
CodeLink Slide 16-Assay Accessory Kit	**310032**

Other materials required for processing

All reagents used in protocol should be molecular biology grade or higher.

- 0.5% NEN blocking reagent (PerkinElmer, FP1020)
- nuclease-free water
- 1 M Tris-HCl, pH 7.6
- 5 M NaCl
- Tween 20
- 20× SSC (Ambion, 9763)
- Isopropanol (to wash reagent container for TNT preparation)
- GenePix 4000B Array Scanner (Axon Instruments) and computer configured for GenePix 4000B Array Scanner or other CodeLink™ validated scanner (see **www.appliedmicroarrays.com** for CodeLink™ validated scanner application notes.)
- centrifuge, 4-15C (Qiagen Corp, 81010 or similar) with appropriate centrifuge plate rotor, 2 × 96 (Qiagen Corp, 81031). For alternative centrifuges, contact Applied Microarrays, Inc. technical support or visit our Web site.
- pipette tips, sterile, RNase-free, and aerosol-resistant
- 1.5-ml microcentrifuge tubes, sterile and RNase-free
- 0.2 µl thin-walled microcentrifuge tubes
- pipettors
- 8-channel electronic pipettor
- microcentrifuge
- thermal cycler
- 46 ºC air incubator
- powder-free gloves
- 50–1200 µl pipet tips
- sterile solution basin
- water bath (90 ºC)

Protocol for hybridization and detection

1
Fragmentation of cRNA

1.1 For each bioarray, bring 1 µg of cRNA (from step 7.6 of CodeLink target preparation protocol) to a volume of 5.3 µl with nuclease-free water in a thin-walled microcentrifuge tube.

1.2 Add 1.3 µl of 5× fragmentation buffer for a total volume of 6.6 µl. Place tube in a thermal cycler and heat for 20 minutes at 94 ºC using the heated lid feature.

1.3 Cool to 0 ºC in the thermal cycler for at least 5 minutes.

> Note: Volumes described here include a 10% volume overage.
>
> Do not exceed 100 µl per tube during fragmentation.

2
Preparation of hybridization reaction mixtures

2.1 Set the temperature of the shaker-incubator to 37 ºC for hybridization. Assemble bioarray tray posts from the CodeLink Shaker Kit onto the incubator platform.

2.2 Transfer the cRNA sample from thin-walled tube to a 1.5-ml microcentrifuge tube.
For each single array within the 16-assay bioarray slide to be processed, prepare 66 µl of hybridization solution by transferring the fragmented cRNA sample into the mixture in the 1.5-ml microcentrifuge tube described below.:

19.8 µl hybridization buffer component A
33 µl hybridization buffer component B
6.6 µl nuclease-free water
6.6 µl fragmented cRNA (from Step 1)
66 µl total volume

> If using multiple Chamber Units, do not prepare more than ~1 ml of hybridization reaction mixture in a single micro-centrifuge tube. Larger volumes will not allow good heat transfer.

2.3 Vortex the solution for 5 seconds at maximum speed. Incubate the hybridization solution at 90 ºC for 5 minutes to denature the cRNA.

2.4 Cool the tube(s) on ice for at least 5 and no more than 30 minutes. Load all bioarrays within 30 minutes of denaturing the cRNA.

3
Loading of reaction mixtures into bioarray chambers

3.1 Set the 12-unit Universal shaker tray on a level surface. Place the 16-assay Chamber Unit(s) into the shaker tray with the openings facing up.

During array chamber loading, take care to avoid cross-contamination.

3.2 Vortex the hybridization reaction mixture for 5 seconds at maximum speed. Centrifuge briefly to gather the liquid at the bottom of the tube. Place the tube back on ice.

3.3 For each bioarray chamber, draw the hybridization reaction mixture (60 µl) into a pipette tip. Place the pipette tip at an angle above the bioarray resting on the edge of the chamber unit. (Fig. 2)

3.4 Expel the liquid into the middle of the chamber, taking care to avoid forming bubbles or splashing into other array chambers.

3.5 After loading, seal the Chamber Unit using the sealing strips and sealing tool as described in Appendix 4.

4
Hybridization

4.1 Align the 12-unit Universal shaker tray notches with the front and back posts fixed to the shaker-incubator platform to place the loaded shaker tray into the shaker-incubator. The Chamber Unit should be facing up.

4.2 Set the shaker speed to 225 rpm and incubate slides for 18–24 hours at 37 ºC. It is critical that arrays used in any form of comparison are hybridized for the same amount of time within the given range.

5
Post-hybridization wash

5.1 Remove the Universal shaker tray from the shaker incubator, and place on a level surface at ambient temperature. Process only 4 Chamber Units of 16-assay slides (64 arrays) at a time per person. Leave the trays in the shaker until ready to process.

5.2 While holding the outside edges of the Chamber Unit firmly, remove the 16-assay Chamber sealing strip by lifting the tab and slowly pulling it back at a 60° angle.

5.3 Use an 8-channel electronic pipettor set on a medium flow rate, and flush each assay chamber three times with 250 µl of 0.75× TNT. Add 250 µl of 0.75× TNT to each assay chamber and seal the entire 16-assay Chamber Unit. Transfer the unit to a 46 ºC oven and incubate for exactly 1 hour; longer incubation time may significantly reduce signal intensities.

Filter all TNT buffer solutions (Appendix 1) through a 0.2-µm filter prior to use.

Prepare sufficient volumes of 1× and 0.75× TNT buffers (Appendix 1) for the entire procedure.

Avoid contact with the probe-containing surface of the microarray throughout the post-hybridization steps.

Do not allow the bioarrays to dry during any of the processing steps.

Clean the shaker incubator platform and the shaker tray with deionized water after each hybridization run.

If processing an odd number of Chamber Units during secondary labelling, prepare the centrifuge counter balance unit for the bioarray drying step.

Caution: Move into step 6.5 (the drying step) immediately because slides may dry non-uniformly in the air, leading to non-uniform signal loss.

Remember to use a centrifuge balance unit.

6.
Detection with streptavidin-dye conjugate

6.1 Remove sealing strip as indicated in step 5.2. Aspirate the 0.75× TNT, ensuring that a fluid layer is kept on the bioarray.

6.2 Add 250 µl of Cy5-Streptavidin for Microarrays for Microarrays working solution to each assay chamber. Place 16-assay Chamber Unit in a dark area at ambient temperature for 30 minutes.

6.3 After the 30 minutes incubation, aspirate the staining solution. Flush each assay chamber three times with 250 µl of 1× TNT. Add 250 µl of 1× TNT to each assay chamber and incubate at ambient temperature for 20 minutes.

6.4 Aspirate the 1× TNT. Add 250 µl of 0.1× SSC/0.05% Tween 20 solution(s) (Appendix 1) into each chamber, immediately invert the 16-assay Chamber Unit(s) and decant liquid to waste.

6.5 Place a paper towel at the bottom of the holder to collect the liquid. Quickly invert each Chamber Unit on the tray. Place the tray into centrifuge bucket. Dry the bioarrays by centrifugation in the Qiagen Sigma 4-15C centrifuge with corresponding bucket rotor (2 × 96-well plate) or similar system using following settings:

speed:	2000 rpm (644 × g)
acceleration:	9
deceleration:	9
time:	3 minutes

6.6 Hold the Chamber Unit with chamber-side away from you. Remove the unit clips with one hand while holding the slide with the other. Peel the slide away from the Chamber and place the dry bioarrays into a light-protected slide box until they are scanned. Bioarrays should be scanned within two days of assay completion.

6.7 Wash the slide drying tray with Alconox™ soap. Rinse thoroughly with deionized water to remove residual soap then air-dry.

7.
Bioarray scanning and analysis

For scanning with a GenePix Array Scanner, use the following steps.
For information on use of alternative scanners with CodeLink™ bioarrays, visit our website, www.appliedmicroarrays.com.

7.1 Turn the scanner on 15 minutes prior to use.

7.2 Slide the cover to the left to expose the slide holder.

7.3 Lift the latch of the slide holder and lift the upper clip.

7.4 While wearing gloves, load the bioarray into the tray with the slide number label side down and closest to the front of the scanner.

7.5 Pull the clip on the left of the slide out and let the bioarray fall into place. Release the clip to put pressure against the bioarray.

7.6 Handle the bioarray by the edges and move the bioarray toward you.

7.7 Lower upper clip and press down on the latch until it clicks.

7.8 Slide the cover to close the slide holder.

7.9 Open the GenePix software and select the following settings:

setting file name:	Expression 635 16UP 5um.gps
wavelength:	635 nm
PMT voltage:	600 V
laser power:	100%
pixel size:	5µm
focus position:	0 µm

7.10 In the Report tab, open the scanning script by clicking **Scan CodeLink Slide**.

7.11 Enter the bioarray serial number and click **Next**. The Experiment and Scan Information interface is displayed.

7.12 Type in the project name, experiment name, and sample name. The username is automatically captured. When opening this interface for the first time, a message box may ask whether to allow an ActiveX interaction to proceed. Click **Yes**.

7.13 Select a setting (.gps) file. The standard setting file is CodeLinkExpr.gps. If a settings file was previously selected, the name and path are displayed under Current Settings File.
To select a new file, click **Browse** under Select New Settings File.
The values for user name and settings file that were entered for a previous bioarray are retained, but may be changed.

Ensure that the slide does not move laterally over the clear gasket; this will damage the array surface.

Keep the bioarrays covered until they are scanned to protect them from dust and light, and handle them only with gloves.

For best results, scan bioarrays within two days of staining.

If bioarrays will be retained for future scanning, they should be stored in a dry box protected from light.

If regions of high background on the bioarray are noted upon scanning, the bioarrays may be rerinsed by repeating steps 6.4 and 6.5. However, not all incidents of high background can be removed by rerinsing.

Refer to the manufacturer's manual for use and maintenance of the scanner (1).

7.14 In the **Load and Scan Slide** screen, the standard TIF file name for the current bioarray is displayed. If desired, the name may be changed.

7.15 Click **Browse** to select the image path or the location where the image files will be stored. If a Security Alert message box is displayed, click **Yes**.

7.16 Click **Scan Slide**. The Image tab will display, and the instrument will perform the scan.

7.17 When complete, the view will return to the Report tab.

7.18 Click **Save Image** to save the scanned image at the appropriate file location.

7.19 Slide the cover to the left and remove the bioarray.

7.20 To scan the next bioarray, click **New Slide** and enter the serial number for the next bioarray. The setting information previously entered will be retained.

7.21 Analyze the image from each bioarray using the CodeLink™ Expression Analysis software.

Appendix 1: Stock solutions

1× **TNT buffer**

0.10 M Tris-HCl, pH 7.6
0.15 M NaCl
0.05% Tween 20

1. Prepare sufficient volumes of 1× TNT buffer for the entire procedure. A single 16-assay bioarray (one slide) utilizes approximately 16 ml of 1× TNT.

2. Rinse a 500 ml reagent container with 100 ml isopropanol. Rinse the reagent container twice with 100 ml of deionized water and completely drain the reagent container.

3. For 250 ml, in a separate container, add the following:

25 ml	1 M Tris-HCl, pH 7.6
7.5 ml	5 M NaCl
125 µl	Tween 20
217.4 ml	deionized water

4. Mix well by swirling. Filter TNT through a 0.2 µm filter and transfer to the isopropanol-rinsed reagent container. This solution can be stored for up to four weeks at ambient temperature.

0.75× TNT buffer

1. Prepare a sufficient volume of 0.75× TNT buffer for the entire procedure. Approximately 16 ml of 0.75× TNT is needed per slide.

2. Add 25 ml of deionized water to 75 ml of 1× TNT buffer per 100 ml of buffer required.

TNB Buffer (0.6 liters)

0.1 M Tris-HCl, pH 7.6
0.15 M NaCl
0.5% NEN blocking reagent (PerkinElmer, FP1020)

1. To a clean 2-liter Erlenmeyer flask with a stir bar, add the following:

522 ml	nuclease-free water
60 ml	1 M Tris-HCl, pH 7.6
18 ml	5 M NaCl

2. On a stir plate heated to 60 ºC, slowly add 3 g of NEN blocking reagent in 0.5 g increments until all 3 g of blocking reagent are dissolved.

3. Turn off heat and continue mixing for 30 minutes. While the solution is still warm, filter through a 0.88 µm filter.

4. Aliquot the TNB buffer into 50-µl tubes and store at -20 ºC. TNB can be stored for 12 weeks at -20 ºC.

5. For use, thaw TNB overnight at 4 ºC. Thawed aliquots can be stored at 4 ºC up to one week.

Cy5-Streptavidin for Microarrays stock solution

1. Add 1 ml of nuclease-free water to the 1 mg bottle of lyophilized Cy5-Streptavidin for Microarrays. This reagent already contains buffer components. To mix, pipette gently up and down or vortex with moderate speed. Do not allow foam to form. Place on ice for 5 minutes. Repeat the mixing three additional times, placing on ice between mixings. Protect this solution from light at all times.

Minimize exposure of Cy5-containing solutions to ambient light.

2. Aliquot the Cy5-Streptavidin for Microarrays solution for the number of bioarrays processed in a typical run. For example, dispense 900 µl aliquots for 3 separate slides (48 arrays).

3. This solution can be stored up to six months (or until stock bottle expiry date) at -20 ºC, protected from light. Once thawed, unused portions of aliquots may be used for up to one week if stored protected from light at 4 °C. Avoid multiple freeze-thaw cycles.

1:500 Cy5-Streptavidin for Microarrays working solution

1. Prior to dilution, centrifuge the thawed Cy5-Streptavidin for Microarrays stock for 1 minutes at ≥ 8000 × g to remove any precipitates.

2. Add 8.8 µl of the Cy5-Streptavidin for Microarrays stock solution to 4.4 ml of filtered TNB buffer for each 16-assay Chamber Unit to be processed. Mix gently by inversion. Use this working solution within 15 minutes of preparation.

0.1× SSC/0.05% Tween 20 (1 liter)

20× SSC (Ambion, 9763)

Tween 20

1. Make sure all solutions are molecular biology grade. Prepare sufficient volume of 0.1× SSC/0.05% Tween buffer for the entire rinsing or re-rinsing procedure. Approximately 4 ml of 0.1X SSC/0.05% Tween buffer is needed for a single slide of 16 arrays.

2. In a clean 500 ml container, add the following:

248.6 ml deionized Water
125 µl Tween 20
1.25 ml 20× SSC buffer

Mix well by swirling. This solution can be stored up to 2 weeks at ambient temperature.

Appendix 2: Bacterial mRNA control use interpretation

Extracting Positive Control Data from the CodeLink Expression Analysis Software

On the Expression Analysis menu in CodeLink™ Expression Analysis v5.0, select Control Probes Report. Select and use the Positive Controls with spots of all quality flags and update the graph. The graph can be easily copied and pasted for record-keeping purposes.

Interpretation

The final mass ratios of the bacterial control mRNAs to the total RNA for the dilutions given are:

1:10 000 000 for *araB, entF, fixB, gnd, hisB* and *leuB*

For a typical mammalian cell, mRNA comprises 1–5% of the total RNA content. If the proportion of mRNA content within total RNA for a particular tissue is unknown, then an estimate of the mass ratios of the bacterial control mRNAs to the target mRNA within the total RNA can be made by assuming a midpoint of this range, or 3% mRNA content. The above total RNA mass ratio then corresponds to an estimated mRNA mass ratio of 1:300 000 for all the bacterial transcripts. The normalized signals from these controls can be graphed, generating sensitivity plots like that shown in Figure 2 below. The noise level for each probe is indicated by the red line and the positive control probe signal above that line shows that the ability to detect signal above noise is at an estimated sensitivity of one copy per cell. Good system performance is indicated if >70% of the positive controls probes exhibit signal above their local noise.

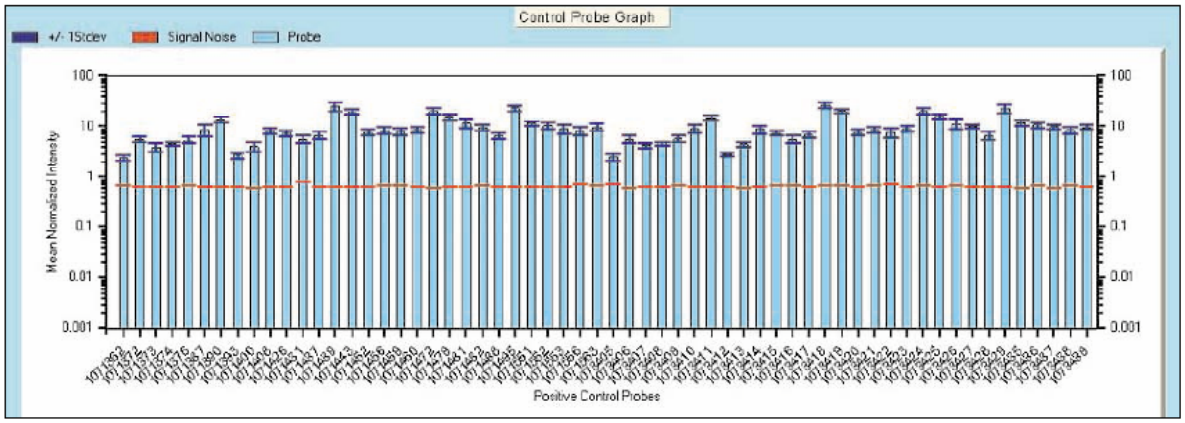


Fig 2. Typical sensitivity plot showing that normalized signal from each bacterial control probe is above noise for the set of control mRNAs spiked at an estimated mass ratio of 1:300 000.

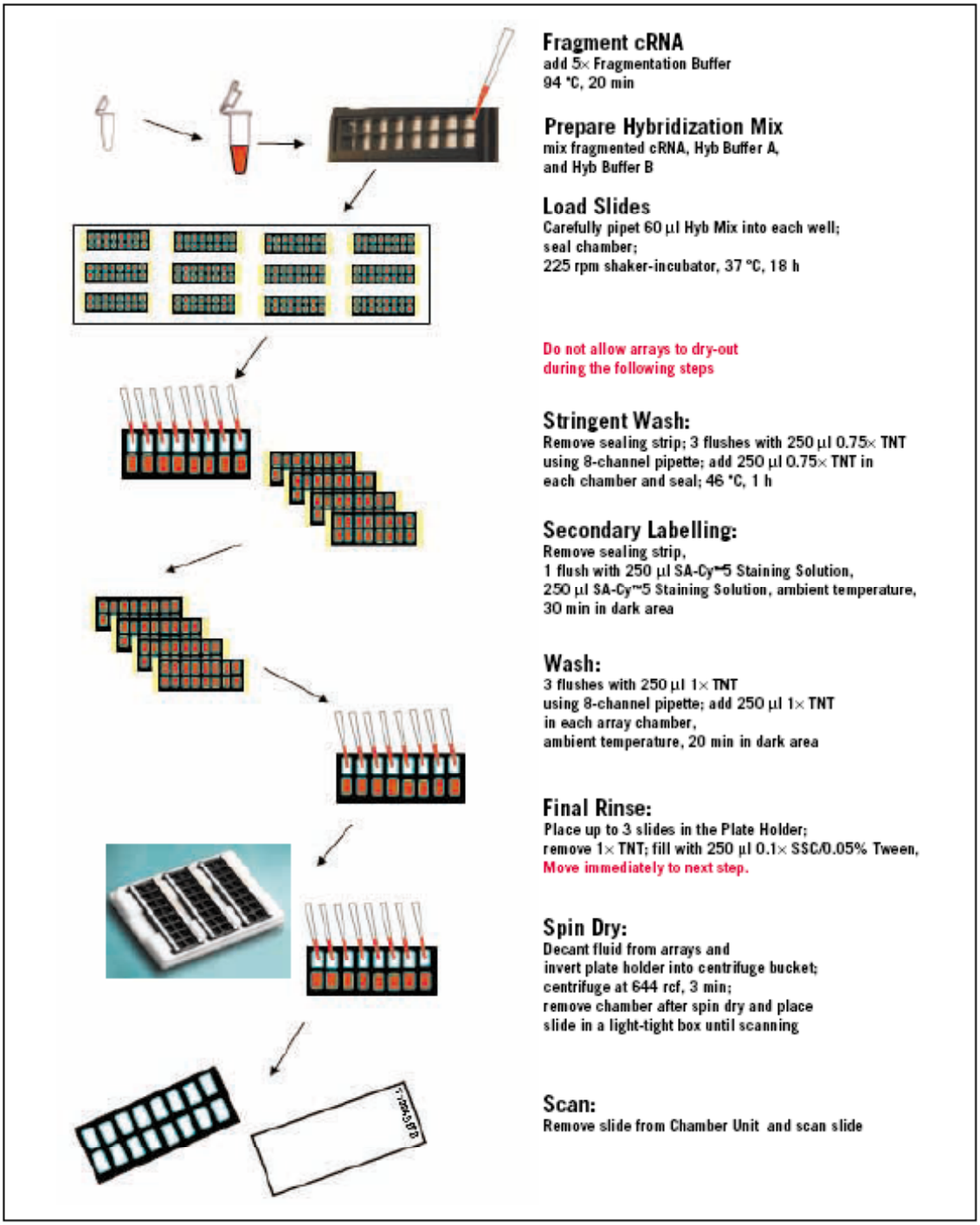
Appendix 3: Sealing the 16-assay Chamber Unit

1. Place the Sealing Strip over the 16-assay Chamber Unit using the sealing tool.

Adhesive will attach firmly at first contact. Use caution to ensure that the Sealing Strip is in the proper orientation before placing it on the chamber. **Do not remove a misaligned Sealing Strip.** Instead, place a second strip to cover any open ports.

Appendix 4: Process flow charts

1. Hybridization and Bioarray Processing



Fragment cRNA
add 5× Fragmentation Buffer
94 °C, 20 min

Prepare Hybridization Mix
mix fragmented cRNA, Hyb Buffer A,
and Hyb Buffer B

Load Slides
Carefully pipet 60 µl Hyb Mix into each well;
seal chamber;
225 rpm shaker-incubator, 37 °C, 18 h

Do not allow arrays to dry-out
during the following steps

Stringent Wash:
Remove sealing strip; 3 flushes with 250 µl 0.75× TNT
using 8-channel pipette; add 250 µl 0.75× TNT in
each chamber and seal; 46 °C, 1 h

Secondary Labelling:
Remove sealing strip,
1 flush with 250 µl SA-Cy™5 Staining Solution,
250 µl SA-Cy™5 Staining Solution, ambient temperature,
30 min in dark area

Wash:
3 flushes with 250 µl 1× TNT
using 8-channel pipette; add 250 µl 1× TNT
in each array chamber,
ambient temperature, 20 min in dark area

Final Rinse:
Place up to 3 slides in the Plate Holder;
remove 1× TNT; fill with 250 µl 0.1× SSC/0.05% Tween,
Move immediately to next step.

Spin Dry:
Decant fluid from arrays and
invert plate holder into centrifuge bucket;
centrifuge at 644 rcf, 3 min;
remove chamber after spin dry and place
slide in a light-tight box until scanning

Scan:
Remove slide from Chamber Unit and scan slide

References

1. *GenePix 4000 User's Guide*, Axon Instruments, Inc., 2500–136 Rev E (2001).

Applied Microarrays, Inc.
7700 S. River Parkway
Tempe, AZ 85284 USA
Email: sales@appliedmicroarrays.com
Phone : (480) 775 6320 FAX: (800) 927 9315
www.appliedmicroarrays.com